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Are *C. elegans* receptors useful targets for drug discovery: Pharmacological comparison of tyramine receptors with high identity from *Caenorhabditis elegans* (TYRA-2) and *Brugia malayi* (Bm4)

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Abstract

The biogenic amine, tyramine (TA), modulates a number of key processes in nematodes and a number of TA-specific receptors have been identified. In the present study we have identified a putative TA receptor (Bm4) in the recently completed *Brugia malayi* genome and compared its pharmacology to its putative *C. elegans* orthologue, TYRA-2, under identical expression and assay conditions. TYRA-2 and Bm4 are the most closely related *C. elegans* and *B. malayi* BA receptors and differ by only 14 aa in the TM regions directly involved in ligand binding. Membranes from HEK-293 cells stably expressing Bm4 exhibited specific, saturable, high-affinity, [³H]LSD and [³H]TA binding with K_ds of 18.1 ± 0.93 nM and 15.1 ± 0.2 nM, respectively. More importantly, both TYRA-2 and Bm4 TA exhibited similar rank orders of potencies for a number of potential tyraminerigic ligands. However, some significant differences were noted. For example, chlorpromazine exhibited an order of magnitude higher affinity for Bm4 than TYRA-2 (pK_is of 7.6 ± 0.2 and 6.49 ± 0.1, respectively). In contrast, TYRA-2 had significantly higher affinity for phenolamine than Bm4. These results highlight the utility of the nearly completed *B. malayi* genome and the importance of using receptors from individual parasitic nematodes for drug discovery.

Keywords

Tyramine receptor; nematode; *Brugia malayi*; *Caenorhabditis elegans*

Introduction

The biogenic amines, tyramine (TA) and octopamine (OA) modulate a number of key processes in nematodes, including pharyngeal pumping, locomotion and egg-laying, suggesting that TA/OA mediated signaling may provide useful targets for anthelmintic development. In general, TA/OA oppose the action of serotonin (5-HT) [1]. For example, in

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the free-living nematode, *Caenorhabditis elegans*, exogenous TA or OA cause hyperactivity, inhibit pharyngeal pumping on bacteria or 5-HT and suppress 5-HT stimulated egg-laying [2–4]. The OA inhibition of pumping appears to involve $G\alpha_o$, as OA inhibits the firing rate of the pharyngeal muscle in wild-type *C. elegans*, but has no effect on *goa-1* mutants defective for $G\alpha_o$ function [5].

Recently TA- and OA-specific signaling pathways have been described in both insects and *C. elegans* [6, 7]. TA and OA are synthesized from tyrosine. Tyrosine decarboxylase (*tdc-1*) converts tyrosine to TA and then tyramine β -hydroxylase (*tbh-1*) hydroxylates TA to form OA [1, 8]. In *C. elegans*, *tdc-1* is expressed in RIC and RIM neurons, and the UV1 and gonadal sheath cells, while *tbh-1* is expressed only in the RIMs and gonadal sheath cells, suggesting that the RICs release only TA and the RIMs release both TA and OA. In *C. elegans*, a role for TA, independent of OA, has been uncovered by the identification of G-protein coupled receptors with much higher affinities for TA than OA and the examination of *tdc-1* and *tbh-1* null mutants that are unable to synthesize TA/OA and OA, respectively). For example, *tdc-1*, but not *tbh-1*, null mutants do not suppress head oscillations in response to anterior touch, suggesting a specific role for TA in this behavior [1]. Three distinct TA receptors have been characterized in *C. elegans*, SER-2, TYRA-2 and TYRA-3 [6, 9; Hapiak et al., unpublished]. Interestingly, these three TA receptors have different pharmacological profiles with different affinities for TA. These TA receptors also are expressed in different tissues, suggesting potentially different physiological roles [6, 9, Hapiak et al., unpublished].

Recently, *C. elegans* has been used as a model to identify signaling pathways in parasitic nematodes because of its completed genome, the availability of signaling mutants and its well defined molecular genetics. Although *C. elegans* is a useful model for the identification and characterization of potential drug targets in core signaling pathways, nematodes exhibit enormous diversity and significant physiological/biochemical/molecular differences between nematode species have been clearly demonstrated. For example, parasitic nematodes appear to have an active nitric oxide synthase and *C. elegans* does not [10, 11]. In contrast, *C. elegans* proteins are probably not good targets for high throughput screening. Because of the enormous variation among species of nematodes, processes in *C. elegans* may not be exactly duplicated in parasitic nematodes and binding constants and regulation may differ significantly among orthologous nematode proteins. On the positive side, all of anthelmintics currently in use appear to function similarly in both *C. elegans* and parasitic nematodes [12].

Are *C. elegans* G-protein coupled receptors similar to their homologues in parasitic nematodes? Interestingly, 5-HT₁-like receptors from *C. elegans* (SER-4) and *Haemonchus contortus* (5-HT_{1Hc}) were recently characterized by different investigators after heterologous expression [13, 14]. Although these two putative 5-HT receptor homologues exhibited markedly different affinities for 5-HT and a number of other ligands, it was not clear whether those differences resulted from true differences in the pharmacologies of the receptors or artefacts of the expression and/or assay systems. For example, 5HT_{1Hc} was expressed in insect Sf9 cells and assayed using [³H]5-HT and SER-4 was assayed after expression in mammalian LCER6 cells using [¹²⁵I]LSD. Therefore, the present study was designed to compare potentially homologous TA receptors from the free-living nematode, *C. elegans* (TYRA-2), and the filarial parasite, *Brugia malayi* (Bm4), under identical expression and assay conditions to examine how these variables might affect ligand-binding and to determine how similarly these two receptors might bind potential tyraminerbic ligands [9].

Materials and Methods

Materials

Dulbeccos Modified Eagles Medium (DMEM) was purchased from Mediatech (Herdon, VA) and fetal bovine serum (FBS) from HyCLONE (Logan, UT). Penicillin/streptomycin, trypsin/EDTA, laminin, geneticin (G418 sulfate) and goat serum were purchased from Sigma (St. Louis, MO). All cell culture plasticware was purchased from Sarstedt (Newton, NC). COS-7 and HEK293t cells were originally ordered from the American Type Culture collection (ATCC) (Rockville, MD). Restriction enzymes were purchased from New England Biolabs (NEB) (Beverly, MA). The [³H]LSD was purchased from Perkin Elmer (Wellesley, MA) and [³H]TA was purchased from American Radiolabeled Chemicals (St Louis, MO). All other ligands were purchased from Sigma. Unifilter™ plates, top sealer™ and microscint cocktails were from Perkin Elmer (Wellesley, MA). cDNA amplification kits were purchased from BD biosciences (San Diego, CA). *B. malayi* total RNA was a gift from David Spiro, TIGR (Washington, DC)

Methods

Mining the TIGR *B. malayi* database for *C. elegans* TYRA-2 homologues—*C. elegans* TYRA-2 protein sequence was used to identify genes encoding potential homologous genes in *B. malayi*. TBLASTN searches of the *B. malayi* WGS_database were performed (<http://tigrblast.tigr.org/er-blast/index.cgi?project=bma1>) using *C. elegans* protein sequences obtained from Wormbase (<http://www.wormbase.org>). TBLASTN searches were performed with TYRA-2. Contigs with high matches to *C. elegans* proteins were run through a modified gene prediction program, Softberry (<http://sun1.softberry.com/berry/phtml?topic=fgenes&group=program&subgroup=gfind>) [15], specific for *B. malayi* intron/exon splice patterns. Individual *B. malayi* genes, from the original contig hit, were translated into protein and motifs characteristic to GPCR identified, e.g., the DRY motif at the end of TM III and the NPxxY motif within TM VII. Proteins with either of these motifs were then compared with the *C. elegans* protein database (www.ncbi.nih.gov/BLAST), to confirm whether the original *C. elegans* TYRA-2 query had the highest identity to the *B. malayi* sequence. ExPasy translate (<http://ca.expasy.org/>) was used to read through predicted exon splice sites of predicted protein sequences lacking either of the conserved DRY or NPxxY motif, to try and find potential exon sequence coding for these motifs within predicted intronic regions.

Screening of 5' and 3' RACE libraries from *B. malayi*—To identify the full length *B. malayi* TYRA-2 homologue, Bm4, RACE libraries were constructed using total RNA isolated from adult *B. malayi*. Two sets of gene-specific primers were designed, primary and nested, (Integrated DNA Technologies, IA) for both 5' and 3' RACE of Bm4, (5' bm4 5' race 1° 5'-GTAAATGATTGGATTTCATTGCTG-3', bm4 5' race nested 5'-CGGTGTCCAACAAATTGC-3', bm4 3' race 1° 5'-GAACCATGCTGCCAATATAC-3', bm4 3' race nested 5'-GCCAATCTTGCCTTCAGTG-3'. The specificities of initial PCR products were tested using nested vector and gene-specific primers. Amplified PCR products were cloned into the TOPO 2.1 vector (Invitrogen Carlsbad, CA) and the DNA sequence determined by MWG (High Point, NC). About 10 to 20 clones were sequenced for each RACE product. Sequence data from both 5' and 3' RACE was compiled and the full length cDNA (flcDNA) sequence compiled (Seqman Program, DNASTAR, Madison WI) and aligned with the corresponding genomic sequence to confirm gene structure (MegAlign, DNASTAR, Madison WI).

Gene Maps—The *C. elegans tyra-2* gene map was created from the EST wormbase database (www.wormbase.org). The *C. briggsae* CbG109562 gene map was compiled using

genefinder prediction from Wormbase. The *B. malayi* Bm4 gene map was compiled from 5' and 3' RACE data.

Protein alignments—Protein alignments were carried out using DNASTar MegAlign. Proteins were first annotated to include only the areas of high identity; specifically, 17 amino acids (aas) prior to the first conserved aas in TM I (N1.50), 10 aas after TM V and before TM VI and 15 aas after TM VII were removed. Annotated protein sequences were uploaded into MegAlign and initially aligned with Clustal W using default parameters and fine-tuned by hand. Files were saved in a Pileup GCG format and uploaded into BOXSHADE (<http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html>) to identify identical (white on black) and similar (black on grey) aas. BOXSHADE.ps files were downloaded and opened in Canvas X for figure construction.

Phylogenetic tree—TA, OA and trace amine-associated receptor (TAAR) protein sequences were downloaded from NCBI and annotated to include primarily the seven transmembrane regions. Annotated sequences were initially aligned using MegAlign in DNASTar with Clustal W using default parameters and fine-tuned by hand (all alignments available on request). Bootstrapping was undertaken in DNASTar (1000 replicates with random seed) and trees were compiled in PAUP. *C. briggsae* (Cb) and *C. elegans* (Ce) protein sequences were downloaded from Wormbase (www.wormbase.org) and all other sequences were from NCBI, accession numbers as follows. Tyramine/Octopamine receptors; *Anopheles gambiae* (AgOA: **EAA06361**; AgTA: **EAA07468.2**), *Aplysia californica* (AcOA: **AAF37686**), *Aplysia kurdai* (AkOA: **AAF28802**), *Apis mellifera* (AmOA: **NP_001011565**AmTA: **CAB76374.1**AmPTA: **XP_394231.2**), *Ascaris suum* (AsTA/OA: **AAS59268**), *Boophilus microplus* (BmiOA: **CAA09335**), *Bombyx mori* (BmoOA: **BAD11157**), *Drosophila melanogaster* [DmOA: **NP_524669**DmOA2 (OCTB1_DROME): **Q9VCZ3**DmOA3 (OCTB3_DROME) **Q4LBB6**DmTA: **ABE73326**DmTA2: **AAK57748.1**], *Heliothis virescens* (HvOA: **CAA64864**), *Locusta migratoria* (LmOA: **CAA49269**), *Lymnaea stagnalis* (LsOA1: **O77408**LsOA2: **O01670**), *Manduca sexta* (MsOA: **ABI33825.1**), *Papilio xuthus* (PxTA: **BAD72869.1**), *Periplaneta americana* (PaOA: **AAP93817**). TAAR; *Homo sapiens* (HsTAAR1: **AAI01826**) and *Mus musculus* (MmTAAR1: **AAK71238**).

Cloning and sequencing Bm4 cDNA—Full length Bm4 cDNA was amplified from a *B. malayi* cDNA RACE library constructed using total RNA isolated from male and female specimens (a kind donation from David Spiro, TIGR, Washington, DC) and the BD SMART™ RACE cDNA Amplification kits (BD Biosciences, CA), using manufacturers protocol. Gene-specific primers were used to amplify full length cDNA via PCR (5' **CCCGGGATGCAAGAGGTGCACATCGAC** 3' **CCGCGGCTATCGCTTTACATACTCTCTTGC**); restriction enzyme sites in italics and predicted start and stop codons in bold face. Amplified PCR products were run out on 1% agarose gels. Bands of correct size were purified and cloned into TOPO 2.1 (Invitrogen) and sequenced by MWG.

Expression of Bm4 in COS-7 and HEK-293 cell lines—Bm4 flcDNA was further cloned into pDisplay™ mammalian expression vector using the restriction enzyme sites 5' *Xma* I and 3' *Sac* II in frame of an N-terminal HA tag (Invitrogen) and reading frame verified by sequencing, MWG. Bm4 constructs were prepared by Eppendorf Maxi prep and transiently transfected into COS-7, NIH-3T3, CHO and HEK-293t or stably in HEK-293 cells. Bm4 constructs were transiently transfected into COS-7, NIH-3T3 CHO and HEK-293t cells at a range of concentrations (2 ng-8 ng) and at a density of ~5×10⁵ cells per 10 cm dish using Lipofectamine 2000 at 1 µg: 3 µl. Temperature shocked cells were moved

to a 30°C incubator 24 hours post-transfection. Cells were harvested ~48 hours post-transfection. Stable cell lines were created in a similar way, but instead of harvesting at 48 hours, the culture medium was changed to contain 800 µg/ml of antibiotic G418 to select for stable clones. Immunofluorescence staining and radioactive binding of membrane fractions approaches were used to select stable colonies.

Immunofluorescence of cell stable expressing Bm4—Cells were grown and fixed on cover slips with Histochoice™. Fixed cells were washed three times with phosphate buffered saline (PBS) and non-specific sites were blocked with 10% heat-inactivated goat serum for 30 mins. Each coverslip was incubated with monoclonal anti-HA Probe (1:2, 000 Santa Cruz Biotechnologies Santa Cruz, CA) for 1 hour at room temperature, washed three times with PBS, and incubated with anti-mouse FITC conjugated antibody (1:50, Jackman Immunoresearch Laboratories Inc, (West Grove, PA) protected from light. Coverslips were washed three times in PBS, mounted in Vectashield hard set mounting media with DAPI (Vector Laboratories Inc, Burlingame, CA) and examined for fluorescence using a Zeiss Axiophot with a FITC filter.

Membrane preparation—Membranes were prepared as described previously [9]. Briefly, cells were first washed in ice-cold PBS and incubated in hypotonic solution [15 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, (PMSF)] for 5 min at 4°C. Harvested cells were centrifuged and the resulting pellet was resuspended and lysed on ice by sonication (10 pulses of 15 seconds at 45 second intervals) and then centrifuged at 200×g for 10 mins. The supernatant was collected and centrifuged at 98,000 g for 45 mins at 4°C. The pellet containing the membrane fraction was resuspended in TEM buffer (50 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 10 mM MgCl₂, 1 mM PMSF) and stored at -80°C.

Radioligand binding assay—Saturation and inhibition binding assays were performed as described previously [9]. Briefly, saturation binding was achieved by incubating 15 µg of membrane protein with [³H]LSD or [³H]TA at a range of concentrations (1 nM – 40 nM) to obtain the B_{max} and K_d. Inhibition assays were performed with various ligands to determine their affinities to displace 10 nM [³H]TA. For both assays total and non-specific binding were determined in the absence and presence of 1000-fold excess of unlabeled LSD or TA, respectively. Receptor binding experiments were incubated at room temperature for 1 hour in restricted light and terminated by filtration through a 96 well micro G/B filter plates (PerkinElmer), previously soaked in 0.3 % polyethyleneimine. Filters were washed three times in ice-cold TEM buffer, dried and the radioactivity retained was measured by liquid scintillation counting. Binding data was analyzed by curve fitting (DeltaGraph, DeltaPoint Inc, 1993). Each experiment was performed in triplicate at least three times. Student t-tests were performed using <http://www.physics.csbsju.edu/stats/t-test.html>.

Results

Cloning and sequence analysis of Bm4

A gene encoding a putative *C. elegans* TYRA-2 homolog, *Bm4*, was identified in the *B. malayi* genome based on the identity of its predicted ORF with to TYRA-2. To isolate the cDNA encoding Bm4, gene-specific primers were designed against the start and stop codons identified from 5' and 3'RACE. Using a *B. malayi* cDNA RACE library as a template, a 1.42-kb fragment was isolated. The predicted amino acid sequence of *Bm4* contains seven putative TM regions and characteristic GPCR motifs: a DRY motif at the end of TM III and NPxxY motif within TM VII [16, 17].

B. malayi gene maps were compiled from the 5' and 3' RACE data and the *B. malayi* genomic sequence database. *Bm4* shares 16 of the 18 intron/exon borders with *tyra-2*, and the predicted *Bm4* protein sequence is 78% identical to TYRA-2, with the highest identity, 83%, in the TM regions (Figure 1). Only 14 amino acids in the TM regions forming the ligand-binding pocket differ between the two receptors: A3.33T and E3.36V in TM III, V5.40I, T5.51L, L5.52F and L5.52I in TM V, L6.37F, T6.45A, V6.46I, A6.49T, T6.53I and L6.57V in TM VI and E7.35W and V7.55I in TM VII (Figure 1).

Identification of Bm4, a putative *C. elegans* TYRA-2 homologue in *B. malayi*

Thirty-four TA or OA receptors from 19 different species were identified in an NCBI search (see Materials and Methods) using TYRA-2. The G-protein coupling of 18 of the 34 receptors is known or strongly suggested through experimentation [18–32]. An unrooted phylogenetic tree of annotated TA/OA receptors using maximal parsimony was compiled using PAUP (Figure 2). Surprisingly, the TA and OA receptors did not cluster according to species, but, instead clustered according to documented G-protein coupling to either $G\alpha_s$ and $G\alpha_{i/o}$. Three main clades are present within the tree; clade I contains all of the characterized $G\alpha_s$ -coupled OA receptors and clade II all of the $G\alpha_{i/o}$ -TA receptors. Bm4 clusters in clade II with TYRA-2, CbP02670 (*C. briggsae* predicted homologue) and *Lymnaea stagnalis* (LsOA2), suggesting that Bm4 is also a $G\alpha_{i/o}$ -coupled TA receptor (Figure 2). Clade III includes DmTA2 and a number of uncharacterised receptors (AmPTA, CeM03F4.3, CbP03587 and Bm5) that are clearly distinct from clades I and II from bootstrap analysis. DmTA2 has recently been identified as a novel TA-specific insect receptor that couples an activation Ca^{++} dependent chloride channels after expression in *Xenopus* oocytes (30).

Multiple alignments of these TA/OA receptors revealed specific aa are conserved in the individual clades (Figure 3). For example, W3.52 and R6.32 are present in all $G\alpha_{i/o}$ -coupled clade II receptors. In contrast, all $G\alpha_s$ -coupled clade I receptors contain L, V, Y, I or C at 3.52 and an K at 6.32. In addition, T2.38 and S3.47 are conserved in all $G\alpha_s$ -coupled clade I receptors, with $G\alpha_{i/o}$ -coupled clade II receptors containing P/Q at 2.38 and G/A at 3.47. In addition, 65 aas are conserved in both TYRA-2 and Bm4, but not in other clade II receptors (Figure 3).

Characterization of Bm4 after heterologous expression in mammalian cells

The Bm4 cDNA was cloned into pDisplayTM and transiently transfected with Lipofectamine 2000TM into a number of mammalian cell lines (NIH-3T3, COS-7, HEK-293 or CHO), resulting in the expression of an N-terminal HA tagged receptor. Unfortunately, expression only reached a maximum of ~7% in each of these lines (as visualized by immunofluorescence to the N-terminal HA tag) and isolated membranes did not exhibit significant saturable [³H]LSD binding. Heat shock 24 hours after transfection, as described for the increased expression of nematode neuropeptide receptors [33], or increasing the amount of transfected DNA (maximum of 8 µg/10 cm plate) did not increase expression. Therefore, stable HEK-293 cell lines expressing Bm4 were prepared as described in methods. Membranes from HEK-293 cells stably expressing Bm4 exhibited saturable, high affinity [³H]LSD binding, with a B_{max} and K_d of 1.79 ± 0.2 pmol/mg and 18.1 ± 0.93 nM, respectively (Figure 4). Most importantly, [³H]LSD binding was inhibited with high affinity by TA (data not shown). As predicted, these isolated membranes also exhibited saturable, high affinity [³H]TA binding with a B_{max} and K_d of 1.15 ± 0.05 pmol/mg and 15 ± 0.2 nM, respectively (Figure 4).

To determine the pharmacological profile of the Bm4 receptor, potential tyraminerigic ligands were tested at varying concentrations for their ability to displace 10 nM [³H]TA in

membranes isolated from HEK-293 cells stably expressing Bm4. The rank order of potency was as follows; TA > chlorpromazine > cyproheptadine = mianserin = DA > synephrine > phentolamine > tolazoline = OA, confirming the identification of Bm4 as a TA receptor (Figure 5 and Table 1).

Comparison of the pharmacological profiles of Bm4 and TYRA-2

To determine whether Bm4 and its putative *C. elegans* orthologue, TYRA-2, bound ligands with similar affinities, the inhibition binding of both receptors was compared under identical expression and assay conditions in membranes isolated from HEK-293 cells expressing the individual receptors. As predicted from the sequence alignments, the pharmacological profiles of Bm4 and TYRA-2 were quite similar (Table 1). However, Bm4 had a significantly higher affinity for chlorpromazine ($pK_{is} = 7.6 \pm 0.2$ and 6.49 ± 0.1 , respectively) and TYRA-2 had a significantly higher affinity for phentolamine ($pK_{is} = 5.81 \pm 0.1$ and 6.4 ± 0.1 , respectively Figure 6). Bm4 also had a significantly greater affinity for DA than OA ($pK_{is} = 6.74 \pm 0.15$ and 5.66 ± 0.12 , respectively), while TYRA-2 had identical affinities for these two ligands ($pK_{is} = 6.22 \pm 0.1$ and 6.19 ± 0.1 , respectively). The rank order of potency for TYRA-2 expressed in HEK-293 cells was as follows: TA > cyproheptadine > chlorpromazine = mianserin > phentolamine > synephrine > OA = DA > tolazoline, mirroring that of TYRA-2 characterized previously after expression in COS-7 cells (Figure 6, Table 2 and Rex et al., 2005).

Discussion

Tyramine and octopamine play important neuromodulatory roles in nematodes. For example, in *C. elegans*, exogenous TA and OA cause hyperactivity and inhibit 5-HT dependent increases in pharyngeal pumping and egg-laying [2, 4]. In general, the effects of TA and OA appear to oppose the action of 5-HT [34]. Unfortunately, given the relative impermeability of the nematode cuticle, the effects of TA and OA on intact nematodes have been measured at very high, unphysiological, concentrations and, given our recent identification of TA-specific receptors in *C. elegans*, it is unclear whether the effects reported previously for OA are not, in fact, mediated by TA receptors. Clearly, TA and OA signaling is complex and is mediated by at least 5 different G-protein coupled receptors expressed at different levels throughout the nervous system [1, 6, 9, 35, 36]. Based on studies in insects and *C. elegans*, TA receptors generally appear to couple to an inhibition of adenylyl cyclase activity through G_{α_o} and OA receptors to a stimulation of adenylyl cyclase activity, presumably through G_{α_s} , although few studies demonstrating direct G-protein coupling are available [1, 22, 27, 37]. Most importantly, OA receptors have been useful targets for pesticide development and, presumably, TA and OA receptors, might serve a similar role in parasitic nematodes. For example, the formamidine pesticides are high affinity OA receptor agonists and activate adenylyl cyclase [37, 38]. These compounds induce behavioral changes, such as hyperactivity, identical to the behavioral changes seen in OA-treated insects [39, 40]. Similarly, cocaine, a naturally occurring insecticide, functions by blocking OA re-uptake and potentiates octopaminergic transmission [41, 42].

In general, parasitic nematodes are difficult to culture and refractory to modern molecular genetics, so that the free-living nematode, *C. elegans* recently has been used as a model to potentially define key signaling pathways. Importantly, all of the commercially available anthelmintics also appear to be effective against *C. elegans* [12, 43]. However, nematodes exhibit enormous diversity and it is not clear whether pathways or receptors identified in *C. elegans* will faithfully translate to parasitic species. Indeed, the recent characterization of putative 5-HT₁-like receptor homologues from *C. elegans* and *H. contortus* suggests that ligand-binding to receptors from the two species might differ significantly [13, 14]. However, since these receptors were characterized under dramatically different expression

and assay conditions it is difficult to make any definitive comparisons. In fact, species differences in the pharmacologies of homologous receptors are not uncommon. For example, the human trace amine associated receptor 1 (HsTAAR1) and its rat homologue (RnTAAR1) have markedly different agonist potencies after expression in AV12-664 cells [44]. Therefore, the present study was designed to characterize the two most identical of the *C. elegans*/*B. malayi* BA receptors, TYRA-2 and Bm4, under identical expression and assay conditions to gain insight into how useful *C. elegans* BA receptors might be as surrogates for drug discovery.

The genome of the filarial parasite is nearly complete. Surprisingly, in contrast to the 15 genes encoding BA receptors in *C. elegans* and its close relative *Caenorhabditis briggsae*, the *B. malayi* genome appears to contain only seven predicted BA receptors, although all of the predicted *B. malayi* receptors appear to have clear homologues in *C. elegans* (Smith et al. unpublished). TA and OA receptors cluster according to predicted G-protein coupling, suggesting that the tree constructed in the present study may be useful for predicting the G-protein coupling of the as yet uncharacterized TA/OA receptors. For example, all G_{α_s} -coupled receptors cluster in clade I and we predict that MsOA, AgOA and CbP03135 are also G_{α_s} -coupled OA receptors. As predicted HsTAAR1, that has high affinity to TA and couples to G_{α_s} , is found in clade I supporting clustering based on coupling and not ligand-specificity [29]. In contrast, all $G_{\alpha_{i/o}}$ characterized receptors fall into clade II and we predict that AsTA/OA, CbP1888, AgTA2, PxTA, BmiOA, LsOA2, Bm4, CbP02670 are $G_{\alpha_{i/o}}$ -coupled TA receptors. Based on an analysis of the sequence alignments, 41 aa are conserved in the TA/OA GPCR family; this number is significantly higher than the 33 aas that are conserved in the 12 human 5-HT receptors reported previously [45]. Interestingly, 65 aas that are conserved in both TYRA-2 and Bm4 are different in other $G_{\alpha_{i/o}}$ clustered receptors, supporting the pharmacological differences between TYRA-2 and SER-2 reported previously [6, 9].

TYRA-2 and Bm4 differ by only 14 amino acids in the transmembrane regions forming the ligand-binding pocket (TM III, V, VI and VII) and both receptors also share the S1xxxxS5 motif in TM V thought to be responsible for TA binding [46]. The pharmacological profile of TYRA-2 expressed in HEK-292 cells mimicked that of TYRA-2 expressed in COS-7 cells published previously [9]. This result is perhaps surprising as pharmacologies of other GPCRs are often affected by cell line and expression level. In contrast, although Bm4 and TYRA-2 exhibited nearly identical affinities for predicted agonists, such as TA and mianserin, they exhibited significantly different affinities for a number of antagonists, most notably, chlorpromazine and phentolamine (Table 1). Chlorpromazine is a relatively non-specific antagonist and binds to DA (D2), 5-HT₁ and 5-HT₂, histamine (H1) and muscarinic (M1/M2) receptors with high affinity. Chlorpromazine interacts with aas in TM II of the γ subunit of the nicotinic acetylcholine receptor (CTL**SIS**xxL* with * indicating aa bound to chlorpromazine), but no studies on the specificity of chlorpromazine binding to GPCRs have been reported [47]. The aas binding chlorpromazine in the nicotinic receptor are spaced about 10.7Å apart in the TM II α -helix, a distance compatible with the crystal structure of chlorpromazine. Phentolamine is a non-selective alpha-adrenergic antagonist whose structure is similar to chlorpromazine. Both chlorpromazine and phentolamine have differential affinities for the two receptors, suggesting that the 14 aa differences between the two receptors ligand-binding TM regions should account for the differences in binding between these two structurally similar ligands (Figure 1).

The physiological role of TYRA-2 in *C. elegans* has yet to be determined. Although GFP fluorescence from *tyra-2::gfp* translational fusions is observed in a number of neurons in the pharynx, nerve ring and tail [9], TA dependent phenotypes have yet to be expressed in *tyra-2*-null worms. The results of the present study demonstrate that even two closely related

nematode BA receptors, differing by only 14 amino acids in the ligand-binding TM regions, still exhibit significant pharmacological differences. These results highlight the utility of a bioinformatics approach for predicting ligand-specificity and coupling of receptors from parasitic nematodes and the importance of using receptors from the parasitic nematode species of interest for drug discovery.

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Figure 1. Gene maps and protein alignment

A, Gene maps were constructed from EST data from Wormbase for *Ce tyra-2* and from 5' and 3' RACE data for *Bm4*. Exons are boxes and intronic sequence is depicted by solid lines. Gray boxes within exons depict sequence coding for TM regions. B, Annotated protein sequences were aligned by MegAlign using Clustal W and sequence analysis by BOXSHADE. White type on black background indicates identical aas; Black type on grey background indicates similar aas. Predicted TM regions are indicated by a solid line above the alignment.

Figure 2. Unrooted phylogenetic tree of Tyramine and Octopamine receptors

Protein sequences were annotated to include primarily the 7 TM regions; specifically the N-termini were deleted including 19 aa before the first conserved aa in TM I (N1.50), the third intracellular loop was deleted 10 aa after TM V and before TM VI and C-termini were removed 15 aa after TM VII. Annotated sequences were initially aligned in DNASTar with Clustal W using default parameters and fine tuned by hand (all alignments available on request). Bootstrapping was undertaken in DNASTar (1000 replicates with random seed) and trees were compiled in PAUP. Tyramine/Octopamine receptors; *Anopheles gambiae* (AgOA, AgTA2.), *Aplysia californica* (AcOA), *Aplysia kurdai* (AkOA), *Apis mellifera* (AmOA, AmTA2, AmPTA), *Ascaris suum* (AsTA/OA), *Boophilus microplus* (BmiOA),

Bombyx mori (BmoOA), *Drosophila melanogaster* [DmOA, DmOA2 (OCTB1_DROME), DmOA3 (OCTB3_DROME), DmTA, DmTA2], *Heliothis virescens* (HvOA), *Locusta migratoria* (LmOA), *Lymnaea stagnalis* (LsOA1, LsOA2), *Manduca sexta* (MsOA), *Papilio xuthus* (PxTA), *Periplaneta americana* (PaOA). Trace amine associated receptors; *Homo sapiens* (HsTAAR1) and *Mus musculus* (MmTAAR1).

Figure 4. Saturation binding for [^3H]LSD and [^3H]Tyramine with membranes expressing Bm4 Membranes prepared from stably expressing HEK293 cells were incubated with [^3H]LSD (A) [^3H]TA or (B) at concentrations ranging from 1 nM–40 nM in the presence or absence of 1000-fold unlabelled LSD or TA, respectively. Specific binding is represented by closed squares and non-specific binding by triangles. Bm4 displays affinity for [^3H]LSD with a K_d of 18.1 ± 0.93 nM and $B_{\max}$ of 1.79 ± 0.2 pmol/ mg and for [^3H]TA a with K_d of 15.1 ± 0.2 nM and $B_{\max}$ of 1.15 ± 0.05 pmol/mg.

Figure 5. Pharmacological profile of Bm4

Membranes of HEK293 cells expressing Bm4 were incubated with 10 nM $[\text{^3H}]\text{TA}$ in the presence of ligands at a range of concentrations. Data are representative of at least three independent experiments performed in triplicate (see Table 1 for summary of pharmacology).

Figure 6. Pharmacological profile of TYRA-2

Membranes of HEK293 cells expressing TYRA-2 were incubated with 10 nM [^3H]TA in the presence of ligands at a range of concentrations. Data are representative of at least three independent experiments performed in triplicate (see Table 1 for summary of pharmacology).

Table 1

Pharmacological profile for Bm4 and TYRA-2

Ligand	HEK-293		COS-7
	Bm4	TYRA-2	TYRA-2
Tyramine (TA)	7.9 ± 0.07 (12.3)	7.7 ± 0.15 (26)	7.4 ± 0.14 (39)
Octopamine (OA)	5.66 ± 0.12 ** (2155)	6.19 ± 0.2 (639)	5.85 ± 0.14 (1412)
Dopamine (DA)	6.74 ± 0.15 ** (179.5)	6.22 ± 0.2 (599)	5.79 ± 0.13 (1621)
Chlorpromazine	7.6 ± 0.2 * (21)	6.49 ± 0.1 (322)	6.6 ± 0.2 (229)
Cyproheptadine	6.8 ± 0.1 (146)	6.96 ± 0.1 (109)	6.59 ± 0.1 (112)
Mianserin	6.78 ± 0.13 (165)	6.56 ± 0.2 (271)	6.62 ± 0.14 (239)
Synephrine	6.04 ± 0.2 (893)	6.11 ± 0.1 (775)	6.07 ± 0.03 (851)
Phentolamine	5.81 ± 0.1 * (1545)	6.4 ± 0.1 (483)	6.1 ± 0.1 (794)
Tolazoline	5.66 ± 0.14 (2150)	5.28 ± 0.13 (5150)	5.53 ± 0.3 (2951)

Membranes of HEK293 cells expressing Bm4 or TYRA-2 were incubated with 10 nM [³H]TA in the presence of ligands at a range of concentrations. pK_is expressed ± SEM and are an average of at least three independent experiments performed in triplicate (K_is in nM in parenthesis). TYRA-2 in COS-7 reproduced from Rex et al, 2005.

* indicates significance difference from TYRA-2 from Student's paired t-test P * < 0.001,

** < 0.05 (95% confidence interval).

Table 2

Comparison of pharmacological profiles of Bm4 and TYRA-2

Receptor	Pharmacological profile
Bm4	TA > Chlo > Cyp = Mia = DA > Syn > Phe > Tol = OA
TYRA-2 (COS-7)	TA > Cyp > Chlo = Mia > Phe > Syn > OA = DA > Tol
TYRA-2 (HEK-293)	TA > Cyp > Chlo = Mia > Phe > Syn > OA = DA > Tol

Competition experiments were performed using 10 nM [^3H]TA, TYRA-2 in COS-7 reproduced from Rex et al, 2005. Chlorpromazine (Chlo), Cyproheptadine (Cyp), Mianserin (Mia), Synephrine (Syn), Phentolamine (Phe), Tolazoline (Tol).